"Split for the cure: VEGF, PDGF-BB and intussusception in therapeutic angiogenesis"

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Key Words: Vascular Endothelial Growth Factor–A (VEGF); Platelet-Derived Growth Factor-BB (PDGF-BB); therapeutic angiogenesis; vascular maturation; pericytes; intussusception

Abbreviations: VEGF, Vascular Endothelial Growth Factor–A, PDGF-BB, Platelet-Derived Growth Factor-BB, Ang-1, angiopoietin-1, CAM, chorioallantoic membrane

Abstract

Therapeutic angiogenesis is an attractive strategy to treat patients suffering from ischemic conditions and Vascular Endothelial Growth Factor-A (VEGF) is the master regulator of blood vessel growth. However, VEGF can induce either normal or aberrant angiogenesis depending on its dose localized in the microenvironment around each producing cell in vivo and on the balanced stimulation of Platelet-Derived Growth Factor-BB (PDGF-BB) signaling, responsible for pericyte recruitment. At the doses required to induce therapeutic benefit, VEGF causes new vascular growth essentially without sprouting, but rather through the alternative process of intussusception, or vascular splitting. Here we briefly review the therapeutic implications of controlling VEGF dose on one hand and pericyte recruitment on the other, as well as the key features of intussusceptive angiogenesis and its regulation.

Introduction

Atherosclerotic cardiovascular diseases, such as peripheral artery disease (PAD) and coronary artery disease (PAD), are the leading cause of death in the Western world [1]. Despite the possibility of pharmacological or surgical therapy, many patients with advanced ischemia are not satisfactorily treated by current procedures and require amputations or progress to heart failure [1, 2]. Therapeutic angiogenesis aims at restoring blood supply through the delivery of growth factors that stimulate the growth of new blood vessels, or their encoding genes, and is an actively investigated strategy to fill this unmet clinical need. Vascular Endothelial Growth Factor-A (VEGF) is the master regulator of vascular growth both in health and disease [3, 4] and is the main molecular target of therapeutic angiogenesis approaches. However, VEGF gene delivery failed to show clear clinical efficacy in several trials [5, 6]. Further, investigation in preclinical models uncovered key issues in VEGF biology that make it challenging to control its therapeutic potential with gene delivery. In fact, uncontrolled VEGF expression produces toxic effects, such as: a) the formation of vascular tumors (hemangiomas) in skeletal muscle, myocardium and other tissues after VEGF gene delivery by genetically modified progenitors [7, 8], plasmid [9] and viral vectors [10]; and b) vascular leakage and tissue edema, leading even to limb loss in preclinical ischemic models [11, 12]. Furthermore, VEGF can interfere with pericyte recruitment [13] and delay maturation of newly formed vessels, which are unstable and regress if the VEGF stimulus is withdrawn before about 4 weeks [14-16].

In order to exploit the therapeutic potential of VEGF, it is crucial to elucidate the cellular and molecular mechanisms of vascular growth induced by its over-expression at therapeutic doses. In fact, the best understood mechanism for vessel growth is sprouting, whereby a tip endothelial cell is generated and migrates towards a VEGF gradient, while neighboring endothelial cells acquire a stalk phenotype and proliferate without migrating to form the new vessel trunk [17]. However, we have recently found that VEGF overexpression at therapeutic doses induces vascular growth in skeletal muscle rather through the alternative mechanism of intussusception, i.e. circumferential enlargement of pre-existing vessels followed by longitudinal splitting [18]. Here we will briefly review the therapeutic implications of controlling VEGF dose on one hand and pericyte recruitment on the other, as well as the key features of intussusceptive angiogenesis and its regulation.

VEGF dose: therapeutic implications

Early attempts at determining the dose-dependent effects of VEGF gene delivery indicated an apparently very narrow therapeutic window in vivo, with increasing vector doses switching rapidly from being inefficient to causing angioma growth [9]. Subsequent studies revealed that spatial distribution of VEGF in the tissue plays a fundamental role in determining its therapeutic window, with significant implications for the design of delivery strategies. In fact VEGF binds to the extracellular matrix (ECM) with different affinity depending on the isoforms, with $VEGF_{120}$ being the most soluble, $VEGF_{188}$ the most binding and $VEGF_{164}$ intermediate [19]. Elegant studies with transgenic mice, selectively expressing physiological levels of only one of the isoforms, showed that vascular networks induced by

$VEGF_{120}$ or $VEGF_{188}$ were both abnormal, with opposite phenotypes of dilated diameters and lack of branching, or very small and hyper-branched vessels, respectively [20]. However, physiological networks were induced by the intermediate $VEGF_{164}$ isoform alone or, intriguingly, by $VEGF_{120}$ and $VEGF_{188}$ together [20], showing the importance of balanced gradients of factor to guide angiogenesis.

We investigated the therapeutic implications of VEGF dose distribution in skeletal muscles by taking advantage of a highly controlled myoblast-based gene delivery platform that we developed over the past decade. In fact, the random integration of retroviral vectors within the genome of transduced myoblasts leads to different expression levels in individual cells. However, monoclonal populations, obtained by isolating single myoblasts from the heterogeneous transduced population, stably and homogeneously produced different amounts of $VEGF_{164}$ from ~5 to ~200 ng/10^6 cells/day. While different amounts of the heterogeneous VEGF-expressing population always resulted in angioma growth, even with a total dose as low as 5 ng/10^6 cells/day, homogeneous expression of specific VEGF levels up to ~60 ng/10^6 cells/day by different clones induced only normal, stable and pericyte-coated capillaries, and angiomas appeared only above a threshold level of ~100 ng/10^6 cells/day [15]. Therefore, VEGF can induce normal or aberrant angiogenesis depending on its amount in the microenvironment around each producing cell in vivo and not simply on the total dose delivered, as different levels remain localized and do not average with each other in tissue.

The control over dose distribution has remarkable functional consequences. In fact, delivery of the same total dose in a murine model of hindlimb ischemia (~60 ng/10^6 cells/day) did not increase flow when this was the average of heterogeneous levels, but fully restored perfusion to non-ischemic levels when it was uniformly distributed in tissue by implanting a monoclonal population [21]. Interestingly, all doses inducing normal angiogenesis caused a similar 2-fold increase in the amount of newly induced vessels, whereas their diameter increased gradually with VEGF levels, always within the range of normal capillaries. This dose-dependent diameter increase was crucial for therapeutic improvement, as functional flow was not increased by lower VEGF levels (~5 ng/10^6 cells/day) that induced the same amount of angiogenesis, but of significantly smaller size [21]. A dose-escalation study of adenoviral VEGF delivery to rabbit skeletal muscle also found that low vector doses caused sprouting of small-size capillaries and higher titers caused vessel enlargement, but functional benefits were achieved only with vascular enlargement [22].

These findings show that control over the microenvironmental distribution of VEGF levels, which is inherently challenging with gene therapy approaches, is critical to realize the therapeutic potential of VEGF. Towards this end, we recently developed a clinically applicable platform to achieve precise control over the dose, distribution and duration of recombinant VEGF delivery in vivo, based on the covalent cross-linking of an engineered VEGF protein into a fibrin hydrogel, similarly to the process of clot formation, and its subsequent release by controlled fibrinolysis in the tissue [23].

Pericytes and vascular maturation

After endothelial activation by VEGF, the second and final stage of physiological angiogenesis is vascular maturation, whereby nascent structures are invested by pericytes, become quiescent and persist indefinitely [24]. Pericytes establish complex regulatory functions and have been shown to promote both vascular stabilization [25], i.e. the persistence of new vessels independently of continuous VEGF stimulation, and vascular normalization, i.e. the prevention of aberrant angiogenesis by excessive VEGF. Here we will focus on the therapeutic implications of pericyte recruitment in the context of normalization and modulation of VEGF dose-dependent effects.

Pericytes, which express PDGF-Receptor-β (PDGFR-β), are recruited by Platelet-Derived Growth Factor-BB (PDGF-BB) produced by the activated tip cells of sprouting capillaries [26]. Mice lacking either the *Pdgfb* or *Pdgfr-beta* genes showed severely impaired pericyte recruitment and vascular abnormalities, with continued endothelial proliferation, microaneurysm formation and bleeding [26], similarly to the effects of deleting the *Pdgfb*

gene specifically in endothelial cells [27]. Pericytes regulate endothelial morphology and function both through cell-to-cell contact and paracrine signals that involve several pathways, such as Transforming-Growth Factor-β (TGF-β) and its receptors, angiopoietins-1 and -2 (Ang-1 and Ang-2) and their receptor Tie2, and the Eph/Ephrin pathway [25, 28]. Targeting of these pathways is a promising approach to modulate the effects of VEGF delivery and to overcome its limitations. Ang-1 has been shown to protect adult vasculature from leakage and to counteract vascular permeability induced by VEGF and inflammation [29], while co-delivery of the Ang-1 gene could reduce VEGF-induced leakage in a rat model of hindlimb ischemia [30] and to promote the maturation of new vessels, which persisted after 4 weeks, but regressed in the presence of VEGF alone [31].

In the context of VEGF over-expression, the dose-dependent transition between normal and aberrant angiogenesis correlates with a loss of normal pericytes [15] and promoting pericyte recruitment by co-delivery of PDGF-BB can modulate the dose-dependent effects of VEGF. In fact, we found that the transition between normal and aberrant angiogenesis is not a fixed property of VEGF dose, but rather depends on the balance between the relative stimulation of VEGF and PDGF-BB signaling and co-delivery of the two factors completely prevented the induction of angioma-like structures regardless of VEGF dose, yielding instead only morphologically normal, mature and functionally perfused capillary networks [32]. However, since both factors are retained in the matrix immediately around expressing cells [19, 33], complete normalization of VEGF-induced angiogenesis required the precise co-localization of VEGF and PDGF-BB gradients in the microenvironment at fixed relative levels, through balanced co-expression from a single bicistronic construct, whereas expression from independent cell sources or separate vectors only had partial effects [32]. Adenoviral co-delivery of VEGF and PDGF-BB from two independent vectors even impaired pericyte recruitment to nascent vasculature [34], whereas similar adenoviral delivery from a single bicistronic vector yielded homogeneous and mature capillary networks [32], further highlighting the importance of the mode of factor delivery for the therapeutic outcomes. These results suggest that balanced co-expression of VEGF and PDGF-BB may overcome the intrinsic limitations of direct gene therapy approaches with VEGF alone, allowing safe and therapeutic angiogenesis despite heterogeneous VEGF levels of expression in vivo.

Intussusceptive angiogenesis

While most of our current understanding of the molecular regulation of vascular growth stems from powerful genetic models of sprouting angiogenesis, it is being increasingly recognized that the alternative process of intussusception, or vascular splitting, plays a significant role in physiological, pathological and therapeutic settings.

Intussusceptive angiogenesis was first described during rat lung development, which entails a massive 35-fold increase in capillary volume during the first 4 postnatal months [35]. Rather than sprouting tips, multiple tiny holes were observed within the rapidly expanding microvessels by vascular corrosion casting and transmission electron microscopy showed that the hole profiles corresponded to slender transcapillary (intraluminal) tissue pillars with diameters of 1-2.5 μm (Fig. 1) [36, 37]. This process of vascular expansion through insertion of tissue pillars into the vessel lumen was named intussusception after the Latin for “growth within itself”. Alternative nomenclature includes “non-sprouting angiogenesis”, “inverse sprouting”, “luminal division” and “splitting angiogenesis”, the latter reflecting the fact that intussusception creates new vascular segments by splitting a solitary microvessel longitudinally into new daughter vessels.

Mechanism of pillar formation and outcomes

Four consecutive steps of pillar formation have been described during intussusception: I) a contact zone between opposing capillary walls is formed (Fig. 2a-b); II) a perforation of the bilayer contact zone is created (Fig. 2c); III) an interstitial tissue pillar core is shaped that is invaded by pericytes and myofibroblasts with deposition of collagen fibrils (Fig. 2d). During this phase the pillars have diameters up to 2.5μm; IV) the pillars increase in girth and could subsequently reshape, fusing with the neighbor pillars and eventually splitting the primary

vessel into two new segments. The last phase does not entail any further change in the pillar structure.

Intussusceptive angiogenesis was subsequently also demonstrated in many tissues and species during both normal and pathological angiogenesis and it appears to be a general phenomenon. For more details, see recent review articles [38, 39].

Intussusception is involved in growth, remodeling and maturation of the vascular plexus, processes that have different morphological and functional outcomes: 1) the repetitive and alternating occurrence of pillars within the capillary bed initially leads to its rapid expansion and an increase in its complexity (intussusceptive microvascular growth); 2) pillars could appear in rows and then elongate and merge to segregate vascular segments that become small arteries and draining veins within the vascular tree (intussusceptive arborization) [40]; 3) pillar formation occurring within the branching points of small arteries and veins can lead to remodeling via optimization of the branching geometry, such that eccentric repetitive pillar formation and fusion results in the exclusion and pruning of vessel branches (intussusceptive pruning) [41].

Blood flow and intussusception

Vascular adaptation to blood flow was postulated almost 150 years ago by Wilhelm Roux in his doctoral thesis [42]. Djonov *et al.* [41] described for the first time the regulation of intissusception by hemodynamic conditions in the chicken choriaoallantoic membrane (CAM). By clamping one of the major arterial side-branches upstream of the investigated area acutely increased blood velocity. The resulting 50–60% surge in flow rate caused many of the vascular bifurcations within the areas of altered hemodynamics to undergo intussusceptive remodeling within 15 to 45 min after clamping. The rapidity of the response indicates that the haemodynamic trigger does not require changes in gene expression. Based on *in vivo* calculations of haemodynamic parameters, 3D computational modeling revealed that transluminal pillars were located in regions of low shear stress <1 dyne cm^{-2} and flow simulations indicated that the pillars were spatially constrained by neighbouring regions of higher shear stress [43]. It should be pointed out that pillar development is caused by increased flow, but occurs in areas characterized by low shear and turbulent flow conditions [41, 43, 44].

VEGF and intussusception

Recent data suggest that, in addition to haemodynamic forces, intussusception is also regulated by growth factors, particularly by VEGF. However, due to a paucity of appropriate experimental models, only scarce data are available on the molecular control of intussusception. In the rapidly developing chick glomeruli and lung, high VEGF levels were associated with the sprouting phase, while VEGF was downregulated during the phase of intussusceptive vascular growth [45, 46]. However, the temporal course of intussusception in the chicken CAM corresponded to VEGF expression in the microvasculature and inhibition of VEGF signaling retarded, but did not abolish, intussusceptive capillary maturation, suggesting that VEGF may not be essential for intussusceptive angiogenesis, but can promote it [47].

Taking advantage of the highly controlled myoblast-based platform for gene delivery to muscle described above, we recently found that over-expression of VEGF, at the doses required to induce therapeutic benefit, causes new vascular growth essentially without sprouting, but rather through an initial circumferential enlargement of pre-existing vessels, followed by transluminal pillar formation and intussusceptive longitudinal splitting [18]. Interestingly, intussusception was the cellular mechanism underlying both normal and aberrant angiogenesis, induced by different VEGF doses. However, high VEGF levels caused a greater initial enlargement than lower ones and, while intussusception was initiated in both cases, pillar formation could not be completed in the presence of larger diameters, leading to a failure to split into normal capillaries and to the continued circumferential growth of affected vascular segments into angioma-like structures [18].

Conclusions

Angiogenesis is a complex and highly regulated process. The design of rational therapeutic approaches to promote angiogenesis requires a thorough understanding of the mechanisms underlying the induction of physiological or aberrant vascular structures under therapeutic conditions, in which intussusception increasingly appears to play a fundamental role. Therefore, it will be key to elucidate the molecular mechanisms controlling intussusception, which are still poorly understood, compared to sprouting. As pericyte recruitment is a promising target to modulate VEGF dose-dependent effects and increase both safety and efficacy of VEGF gene delivery, it remains to be determined whether pericytes can control intussusception and through which molecular pathways.

Funding

This work was supported by grants of the Swiss Heart Foundation to A.B and to R.G.B. and of the Swiss National Science Foundation to A.B. (310030_143898).

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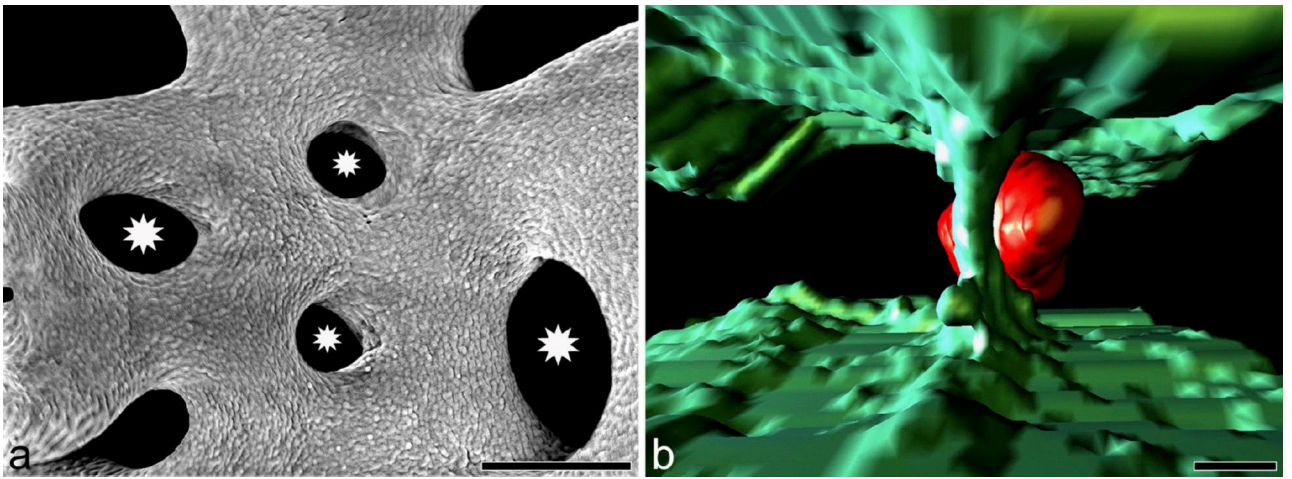
Figure Legends

Figure 1. Morphology of transluminal pillars. (a) Corrosion cast of blood vessels in which newly formed transluminal pillars appear as small holes (stars). (b) 3D reconstruction of a chick CAM transluminal pillar (displayed as hole in a): an erythrocyte (red) is represented behind the pillar (green). Size bars = 5µm. (Reproduced from [48]) and [40]).

Figure 2. Morphogenic events during intussusception. (a-d) 3D scheme of transluminal pillar formation, the hallmark of intussusceptive angiogenesis. (a'–d') 2D representation of the events in a–d. Endothelial cells (EC) on the opposite sides of a capillary protrude into its lumen until they make contact with each other. Once established, this contact is maintained by the formation of inter-endothelial junctions and reorganized so that the endothelial bilayer is perforated centrally. Endothelial cells then retract and the newly formed pillar increases in girth after being invaded by fibroblasts (Fb) and pericytes (Pr), which lay down collagen fibrils (Co). BM = basement membrane. (Reproduced from [49] and [48].


a
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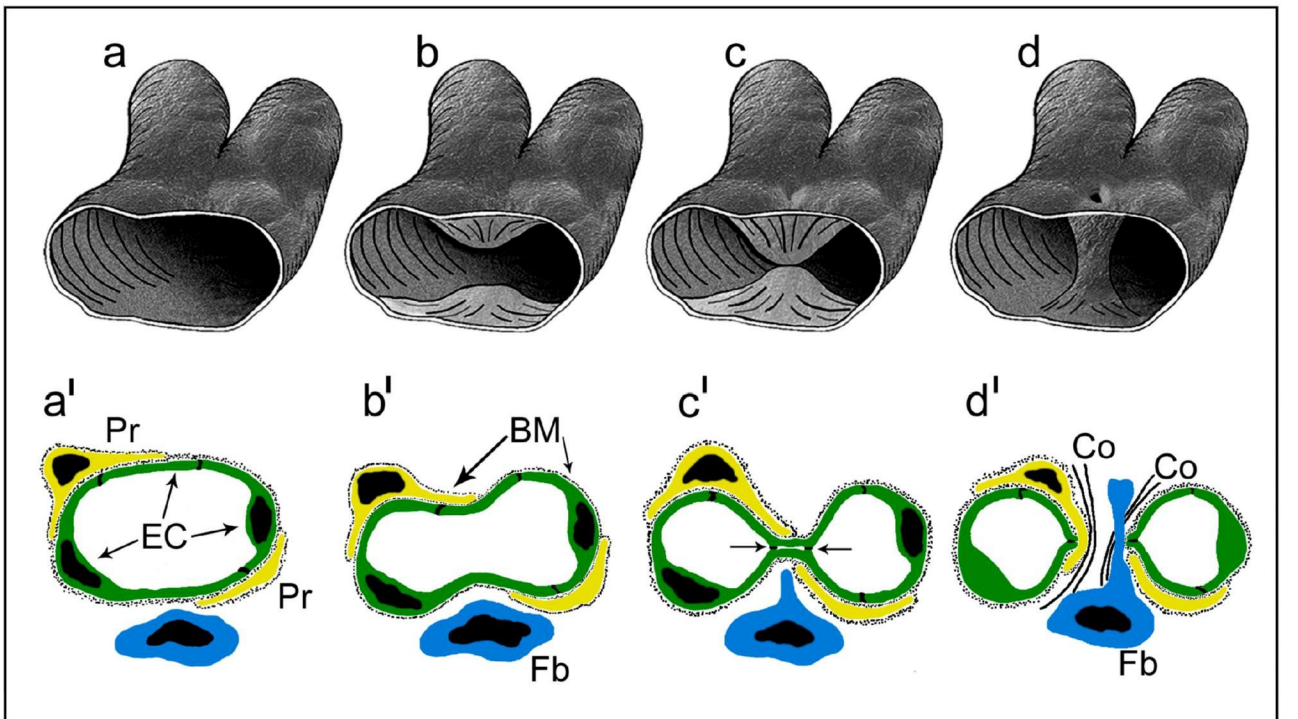
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